Published in final edited form as:

Biomaterials. 2018 December ; 185: 97–105. doi:10.1016/j.biomaterials.2018.09.005.

Mucus-penetrating budesonide nanosuspension enema for local treatment of inflammatory bowel disease

Abhijit A. Date^{#1,2,□}, Gilad Halpert^{#1,2}, Taarika Babu^{1,3}, Jairo Ortiz^{1,2}, Pranjali Kanvinde¹, Peter Dimitrion¹, Janani Narayan^{1,5}, Hannah Zierden^{1,5}, Kalpana Betageri^{1,6}, Olivia Musmanno^{1,6}, Helen Wiegand^{1,6}, Xinglu Huang^{1,2}, Sanjeev Gumber⁴, Justin Hanes^{1,2,3,5,6,*}, and Laura M. Ensign^{1,2,3,5,6,*}

¹The Center for Nanomedicine, The Wilmer Eye Institute, Johns Hopkins University School of Medicine, 400 N Broadway, Baltimore, MD 21231, USA;

²Department of Ophthalmology, The Wilmer Eye Institute, Johns Hopkins University School of Medicine, 400 N. Broadway, Baltimore, MD 21231, USA.

³Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.

⁴Division of Pathology, Yerkes National Primate Research Center, Atlanta, GA 30322, USA.

⁵Department of Chemical and Biomolecular Engineering, Johns Hopkins University, 3400 N. Charles Street, Baltimore, MD 21218, USA.

⁶Department of Biomedical Engineering, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA.

These authors contributed equally to this work.

Abstract

Inflammatory bowel disease (IBD) is a chronic inflammatory gastrointestinal disorder that affects more than 1 million individuals in the USA. Local therapy with enema formulations, such as micronized budesonide (Entocort[®]), is a common strategy for treating patients with distally active

*Co-corresponding authors: Laura M. Ensign, The Center for Nanomedicine, The Wilmer Eye Institute, Johns Hopkins University School of Medicine, 400 N Broadway, Baltimore, MD 21231, USA. l.ensign@jhmi.edu. Phone: 410-614-9854, Fax: 443-287-7922. Justin Hanes, The Center for Nanomedicine, The Wilmer Eye Institute, Johns Hopkins University School of Medicine, 400 N Broadway, Baltimore, MD 21231, USA. hanes@jhmi.edu. Phone: 443-287-7921, Fax: 443-287-7922.

□Current Address: The Daniel K. Inouye College of Pharmacy, University of Hawaii Hilo, 200 W. Kawili Street, Hilo, HI 96720

Declaration of Interest

The mucus-penetrating particle technology is licensed and in clinical development for ocular indications by Kala Pharmaceuticals. J.H. is a founder of Kala Pharmaceuticals and serves as a consultant. J.H. and Johns Hopkins own company stock. Under a licensing agreement between Kala Pharmaceuticals and the Johns Hopkins University, L.E., J.H., and the University are entitled to royalty distributions related to the technology. These arrangements have been reviewed and approved by the Johns Hopkins University in accordance with its conflict of interest policies.

Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

IBD. However, we hypothesize that micronized particulates are too large to effectively penetrate colorectal mucus, limiting the extent of drug delivery to affected tissues prior to clearance. Here, we describe the development of a budesonide nanosuspension (NS) with the appropriate surface coating and size to enhance penetration of colorectal mucus and ulcerated colorectal tissues. We demonstrate that model fluorescent polystyrene (PS) particles ~200 nm in size with a muco-inert Pluronic F127 coating provide enhanced mucosal distribution and tissue penetration in mice with trinitrobenzenesulfonic acid (TNBS)-induced IBD compared to model 2 μ m PS particles coated with polyvinylpyrrolidone (PVP), the stabilizer used in the clinical micronized budesonide formulation. We then used a wet-milling process to develop a budesonide NS formulation with a muco-inert Pluronic F127 coating (particle size ~230 nm), as well as a budesonide microsuspension (MS) stabilized with PVP (particle size ~2 μ m). Using an acute TNBS mouse model of IBD, we show that daily budesonide NS enema treatment resulted in a significant reduction in the macroscopic (decreased colon weight) and microscopic (histology score) symptoms of IBD compared to untreated controls or mice treated daily with the budesonide MS enema. Further, we show that the budesonide NS enema treated mice had a significantly reduced number of inflammatory macrophages and IL- β producing CD11b⁺ cells in colon tissue compared to untreated controls or mice treated with the budesonide MS enema. We conclude that the nano-size and muco-inert coating allowed for enhanced local delivery of budesonide, and thus, a more significant impact on local colorectal tissue inflammation.

Keywords

Wet-milling; colitis; Pluronic F127; budesonide; trinitrobenzenesulfonic acid (TNBS)

Introduction

Inflammatory bowel disease (IBD) is a term that refers to chronic inflammatory diseases that affect the gastrointestinal tract, further divided into ulcerative colitis (UC) and Crohn's disease (CD). IBD is generally characterized by immune system dysregulation, mucosal inflammation, and impaired integrity of the epithelial barrier [1]. Anti-inflammatory drugs, such as steroids, are commonly used to treat IBD symptoms, repair mucosal tissues, and induce remission. The current frontline steroid therapy is budesonide, which has low bioavailability and undergoes extensive first-pass metabolism when taken orally, but is considered to be more effective than other common steroids when dosed topically [2, 3]. For two-thirds of UC patients and for patients with more diffuse disease that also affects the distal colorectum, topical enema therapy is used [4, 5]. While enema products are considered more efficacious and associated with fewer side effects than oral formulations in treating disease in the distal colorectum, substantial room for improvement remains. Although more effective than placebo, rectal budesonide products have provided <40% remission after 6–8 weeks of daily treatment in clinical studies [6, 7].

To achieve effective topical treatment for inflammation in the colorectum, sustained, therapeutic levels of drug must be delivered to the affected tissues and cells. Although the colorectum is relatively accessible by enema administration, additional barriers to effective local drug delivery exist. The mucus layers coating the colorectal epithelial surface are a

major barrier that limits colorectal drug distribution and absorption into the local tissue [8, 9]. Frontline enema formulations for a water insoluble drug like budesonide contain micronized drug particles [10, 11]. However, we found that microparticles are too large to penetrate the protective mucus mesh lining the colorectum and, thus, are sterically restricted from reaching the epithelial surface [9, 12], facilitating rapid clearance [8]. In contrast, we found that nanoparticles that are both (i) small enough to pass through the pores in the mucus mesh, and (ii) non-adhesive to mucus (mucus-penetrating particles, MPP), provide rapid, highly uniform epithelial distribution in the colorectum following enema administration in a hypotonic enema vehicle [9, 13]. Hypotonic vehicles cause rapid, osmosis-driven, advective delivery of non-adhesive MPP through mucus, and right up against the epithelial surface [14, 15]. Further, MPP administered in a hypotonic enema vehicle entered ulcerated tissues in the colorectum in a mouse model of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced IBD more efficiently than microparticles or nanoparticles that were adhesive to mucus [9].

Here, we describe the design of budesonide nanosuspensions (NS) containing drug particles of the appropriate size and formulated with muco-inert coatings (Pluronic F127) for improved mucosal distribution and tissue penetration in IBD. We demonstrate that budesonide NS provide improved colorectal tissue levels of budesonide with minimal systemic exposure compared to a budesonide microsuspension (MS) formulated with the same stabilizer as the clinical product (polyvinylpyrrolidone (PVP)). We further compare the budesonide formulations for efficacy in the TNBS-induced mouse model of IBD, because (i) the inflammatory injury is localized to the colorectum, which is optimal for enema delivery, and (ii) budesonide was previously shown to be efficacious in the TNBS mouse model of IBD [16–18]. The improved colorectal drug delivery and efficacy provided by the muco-inert budesonide NS suggest the potential for clinical utility.

Materials and Methods

Materials

Budesonide (99% purity), 5% w/v 2,4,6-trinitrobenzenesulfonic acid (TNBS) solution, sodium azide, dimethyl sulfoxide (DMSO), 1,4-Dithiothreitol (DTT), phosphate buffered saline (PBS), lipopolysaccharide (LPS), collagenase type VIII, and DNase-I were purchased from Sigma-Aldrich (St. Louis, MO). Polyvinylpyrrolidone K30 (PVP) was purchased from TCI Chemicals (Portland, OR). Zirconium oxide beads (0.15 mm, 0.5 mm and 1.0 mm in diameter) were purchased from Next Advance (Averill Park, NY). Carboxylate-functionalized fluorescent polystyrene (PS) particles (200 nm red and 2 μ m yellow-green) were purchased from Molecular Probes (Eugene, OR). Sterile cell strainers (100 μ m) were purchased from Fisher Scientific (Hampton, NH). Pluronic F127 (Poloxamer 407) was obtained as a free sample from BASF corporation (Tarrytown, NY). Brefeldin A and fluorescent antibodies were obtained from eBioscience (San Diego, CA) or Biolegend (San Diego, CA).

2,4,6-trinitrobenzenesulfonic acid (TNBS) model

All experimental procedures were approved by the Johns Hopkins Animal Care and Use Committee. Male 5–6 week old Balb/c mice were purchased from Harlan (Indianapolis, IN) and acclimated in the animal facility for one week. In our experience, reducing the presence and passage of pellets during TNBS model induction minimizes variability in disease severity. Mice were starved for 6 h and then received a 200 μ L saline enema using a flexible feeding tube to clear the distal 3–4 cm of the colorectum. After 10 min, 65 μ L of TNBS (2.5% TNBS in 50% ethanol) was administered colorectally using a Wiretrol with the mice under isoflurane anesthesia to minimize back flow (Day 0, D0). These procedures resulted in reliable weight reduction (average ~10–12% for each experimental group) within 24 h after TNBS administration (Day 1, D1). The few mice that did not exhibit at least 7% weight reduction 24 h after TNBS administration were excluded, and the remaining mice were grouped to ensure similar average weight loss.

Model particle distribution

Stock (2% w/v) fluorescent 200 nm or 2 μ m PS particles were diluted 10-fold with 2% (w/v) Pluronic F127 solution in water (200 nm/2% F127 and 2 μ m/2% F127) or with 1% (w/v) PVP solution in water (200 nm/1% PVP and 2 μ m/1% PVP) and stored in the fridge overnight. We previously demonstrated that uncoated PS particles 100–500 nm in size distributed poorly in the colons of healthy mice and mice with TNBS-induced colitis [9], and thus did not repeat the uncoated control here. Mice in the “healthy” group did not receive any pretreatments. For the “TNBS” group, TNBS colitis was induced as described above (average weight loss ~10%). Fifty μ L of particles was administered intra-rectally. After 15 min, the distal 4 cm of the colon was excised and cut into 1 cm portions that were embedded, frozen, sectioned, and stained as described [9]. The sections were imaged at different magnifications using a Zeiss LSM 510 Meta confocal microscope.

Budesonide particle formulation and characterization

For the microsuspension (MS), budesonide (5 mg) and 1 ml of 1% (w/v) PVP solution in water were vortexed at 1200 rpm for 5 min. For the nanosuspension, wet bead-milling was performed using a TissueLyser LT (Qiagen Inc, Germantown, MD). The diameter of zirconium oxide beads, the mass ratio of beads to drug, the Pluronic concentration, and the bead-milling time were optimized to obtain a budesonide particle size of ~200 nm. In the final formulation, budesonide (10 mg) and zirconium oxide beads were added to 1 ml of 2% (w/v) Pluronic F127 milled at a speed of 3000 oscillations per min (maximum oscillation speed) in a cold room to dissipate heat. The milling beads were then separated by passing the suspension through a 100 μ m cell strainer. Particle size, polydispersity index (PDI), and surface charge (ζ -potential) of MS and NS formulations were measured using a Malvern Zetasizer Nano ZS (173° scattering angle). MS and NS were diluted 10-fold in ultrapure water for sizing and 40-fold in 10 mM NaCl (pH 7) for ζ -potential measurements. Images of the NS were obtained using a Hitachi H7600 transmission electron microscope.

Evaluation of in vitro activity

Mesenteric lymph nodes (MLN) and spleen were isolated from 7 week old C57BL/6 male mice. Single-cell suspensions of MLN and splenocytes were prepared as described [19]. 200,000 MLN cells or splenocytes/well were seeded in 96 plate wells and activated with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml), ionomycin (500 ng/ml), and LPS (1 µg/ml) for 5 h in the presence or absence of 1 µM of budesonide MS or NS. Brefeldin A was added to each well after 1 h of activation in order to enhance the detection of intracellular cytokines by flow cytometry. RAW 264.7 (murine macrophage cell line, ATCC) cells were cultured at 37°C in humidified atmosphere with 5% CO₂ using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) heat-inactivated FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. For all experiments, 0.5 million cells/well were seeded overnight in 6-well plates. Then, the medium was replaced and cells were incubated for 5 h with 500 ng/ml LPS concomitantly with or without 1 µM of budesonide solution (0.02% DMSO in PBS), 1 µM budesonide MS, or 1 µM budesonide NS. Then, supernatants were removed, cells were washed with PBS and transferred into 96-well plates. Brefeldin A was added for 3 h and the cells were stained with relevant antibodies using flow cytometer (Table S1).

Efficacy experiment

TNBS colitis was induced as described above. Starting on D1, mice received a 100 µL enema containing either 0.5 mg/mL budesonide NS, 0.5 mg/mL budesonide MS, or PBS (untreated control). On Day 2 (D2) and Day 3 (D3), mice were weighed and given enema treatments for a total of 3 daily treatments. Twenty-four hours after the final treatment (Day 4, D4), mice were weighed, and colorectal tissues were obtained. A standard length of 4 cm of colorectal tissue were obtained from each mouse and weighed. Alternatively, the whole colon was used for histology or for isolation of immune cells from the lamina propria (LP).

Histology

Whole colons were prepared using a "sandwich roll" technique, fixed in 10% neutral buffered formalin for at least 24 h, and then transferred to 70% ethanol for at least 24 h. JHU Reference Histology lab performed paraffin embedding, sectioning, and hematoxylin and eosin (H&E) staining. Tissues were scored by a trained veterinary pathologist in a blinded fashion using the scoring system in Table S2. The overall score was the sum of individual scores.

Isolation of colon lamina propia (LP) cells

Colonic LP cells were isolated as described previously [20, 21], counted, and stained with relevant antibodies for flow cytometry analysis (Table S1). For intracellular staining, LP cells were activated with PMA (50 ng/ml) and Ionomycin (500 ng/ml) for 4 h. Brefeldin A was added after 1 h of activation.

Flow Cytometry Analysis

The antibodies used to detect relevant immune cells and intracellular cytokines from RAW264.7 cells, MLN/spleen cells and colonic LP cells are described in Table S1. Viability,

surface and Intracellular staining were performed according to regular standard protocols established by eBioscience (San Diego, CA). All cells were analyzed using a SONY SH800 Cell sorter and FlowJo software.

Statistics

The Student's *t*-test (two-tailed, unequal variance) was used to compare differences between two experimental groups. To compare multiple groups, one-way ANOVA with the Tukey post hoc test was used. For histological scoring, the non-parametric Kruskal-Wallis with Dunn's post hoc test was used. All values are presented as the average $\pm$ the standard error of the mean (SEM) unless otherwise indicated. P values <0.05 were considered statistically significant for all data.

Results

Colorectal distribution of model fluorescent particles

Fluorescent PS particles of the appropriate size and surface stabilizers (Table S3) to serve as model probes for the budesonide NS and MS were intrarectally administered to both healthy mice and mice with TNBS-induced colitis. The lack of colorectal folds and presence of epithelial damage was immediately apparent in the mice with TNBS-induced colitis. Despite this, the 2 μm /1% PVP particles were largely dispersed in the lumen in the TNBS mice (Fig. 1a), similarly restricted from reaching much of the epithelium as in the colons of healthy mice (Fig. 1c). Similar trends were observed for 2 μm /2% F127 particles in the colons of TNBS mice (Fig. S1A) and healthy mice (Fig. S1C). In contrast, 200 nm/2% F127 particles distributed throughout the colorectal lumen in the TNBS mice, including penetrating into ulcerated regions of the tissue (Fig. 1b). In healthy mice, 200 nm/2% F127 particles were more uniformly distributed across the colorectal epithelium, as was previously demonstrated for similarly sized PS nanoparticles with covalently grafted polyethylene glycol (PEG) on the surface (PS-PEG) administered in a hypotonic enema [9]. In contrast, the distribution of 200 nm/1% PVP particles was restricted similarly to the 2 μm particle formulations in both TNBS mice (Fig. S1B) and healthy mice (Fig. S1D). To confirm the apparent adhesive interactions of PVP coated particles and the steric interactions of the larger 2 μm particles in mucus, we tracked the motions of the model coated particles in undiluted human cervicovaginal mucus (CVM), which is convenient to collect fresh and undiluted in large quantities. We confirmed that the ensemble averaged mean squared displacement ($\langle\text{MSD}\rangle$) of 200 nm/2% F127 particles was similar to that of 200 nm PS-PEG particles, whereas the 200 nm/1% PVP particles were largely immobilized in human CVM similar to the unmodified 200 nm PS particles (Fig. S2A). The 2 μm /1% PVP particles were immobilized in human CVM similar to unmodified 2 μm PS particles (Fig. S2B). In comparison, the 2 μm /2% F127 particles showed some mobility in human CVM, but the $\langle\text{MSD}\rangle$ was reduced ~ 100 -fold compared the estimated unrestricted diffusion in pure water (W), suggesting the particles were mostly sterically trapped (Fig. S2B). Together, these results suggest that using both a smaller particle size and a mucoinert coating could provide improved drug delivery to the colorectum.

Development and characterization of budesonide MS and NS

We formulated a budesonide MS using the same stabilizer in Entocort®, PVP. The average particle size was $2.5 \pm 1.25 \mu\text{m}$. For the budesonide NS, we optimized various parameters involved in the wet-milling process using the TissueLyser, including milling bead size (Fig. 2a), the ratio of mass of drug to mass of beads (Fig. 2b), the concentration of Pluronic F127 (Fig. 2c) and milling time (Fig. 2d) to obtain an F127-coated budesonide NS formulation with the appropriate size and surface properties for effective penetration through colorectal mucus (Table S3) [9, 22, 23]. TEM images further confirmed the size of budesonide NS (Fig. 2e). Although the MS and NS were made fresh daily for enema administration, we evaluated the stability of each formulation at room temperature (RT) and 4°C. We generally observed that the size (Fig. S3A) and polydispersity index (PDI) (Fig. S3B) of the NS was relatively stable over 28 days in both storage conditions. As expected, the size and PDI fluctuated more for the MS over 28 days of storage. There were minor fluctuations in the ζ -potential for both the MS and NS (Fig. S3C).

In vitro anti-inflammatory activity of budesonide MS and NS using murine MLN

In order to confirm that processing does not impact the inherent activity of budesonide, we evaluated the *in vitro* anti-inflammatory activity of the budesonide solution (SOL), NS and MS using murine macrophages (RAW264.7 cells). Flow cytometry analysis showed that all budesonide treatments significantly reduced the pro-form of IL-1 β in the LPS-activated RAW264.7 cells, indicating that budesonide SOL, MS as well as NS exerted similar anti-inflammatory effects *in vitro* (Fig. 2f). Further, budesonide MS as well as NS also significantly reduced the levels of inflammatory cytokines TNF α and IFN γ in activated MLN and splenocytes (Fig. S4).

Efficacy in the TNBS colitis model

We next evaluated daily budesonide enema treatment for efficacy in an acute TNBS-induced colitis model. After the first enema treatment on D1, untreated mice (TNBS) continued to lose weight on D2 ($-16.2 \pm 1\%$), whereas the average loss in body weight in the budesonide MS (TNBS + MS) and NS (TNBS + NS) group remained similar ($-12.0 \pm 1.1\%$ and $-13.0 \pm 1.1\%$ respectively). By D3, the average weight loss in the NS group ($-9.5 \pm 1.6\%$) and MS group ($-9.2 \pm 1.4\%$) was significantly less than the average weight loss in the control group ($-17.0\% \pm 1.8\%$). Twenty-four hours after the third treatment on D4, the average weight loss for the NS ($-5.5 \pm 1.3\%$) and MS ($-6.4 \pm 1.5\%$) groups were similar, but significantly lower ($P < 0.01$) compared to the untreated group (Fig. 3a). Importantly, although the overall recovery in weight loss was similar in the NS and MS groups, the average colon weight in the NS group ($107.8 \pm 3.2 \text{ mg}$) was significantly decreased compared to the untreated group ($131.9 \pm 6.9 \text{ mg}$), whereas the MS treated group was statistically indistinguishable compared to the untreated group (Fig. 3b). We further confirmed the improved local tissue effect of the budesonide NS with histopathological analysis of colon tissue. Colorectal tissues from the untreated group showed classic signs of TNBS-induced colitis, such as infiltration of immune cells, loss of epithelial barrier, ulcerations, and atrophy and loss of crypt structure (Fig. 3c/d). Of note, only mice treated with the budesonide NS had a significant reduction in microscopic symptoms of IBD,

whereas colorectal tissues from the mice treated with budesonide MS were indistinguishable from the untreated mice (Fig. 3c/d).

Evaluation of immune cells

To further understand the increased efficacy of the budesonide NS in attenuating local colorectal inflammation, we sought to characterize colon tissue immune cells. We isolated viable colonic LP immune cells (CD45+ LIVE) and used flow cytometry analysis to identify neutrophils (CD45+Ly6G+) and inflammatory macrophages (Ly6C^{high}CD11b+). Both the budesonide MS and NS treatments caused significant reductions ($P<0.01$) in the infiltration of neutrophils into the colon, though the NS trended toward the larger reduction (Figure 4A). Only the budesonide NS treatment provided a significant reduction ($P<0.05$) in the infiltration of inflammatory macrophages that are involved in the progression of IBD (Fig. 4b) and significant reduction in the production of the pro-form of IL-1 β from CD11b+ cells (Fig. 4c). We found that the budesonide MS and NS similarly reduced the presence of adaptive immune cells, including CD4+ T cells, CD8+ T cells, and B cells (Fig. S5).

Discussion

Although oral administration is often considered the most acceptable mode of drug administration, topical drug administration results in much higher local drug levels with lower doses, as well as reduced systemic drug exposure. This is particularly true for IBD drugs like budesonide, which undergoes extensive first-pass metabolism, and has undesirable systemic side effects [2, 3]. Topical enema therapy is more effective for IBD patients with disease affecting only the distal colorectum, and even for patients with additional diffuse active disease, prescribing both an enema and oral medication is more effective than oral therapy alone [5, 24]. Numerous efforts for formulating nanoparticles that can be dosed orally, but transit to the colorectum to deliver drug locally, have been described [25, 26]. There are some limitations to this type of approach, which typically requires the use of an enteric polymer coating to minimize drug release in other parts of the GI tract. The pH changes throughout the GI tract are an attractive attribute for using pH to modulate particle degradation and drug release, but there has been preclinical and clinical evidence demonstrating interpatient variability that leads to premature degradation, or no degradation at all [27, 28]. Particularly in diseased states like IBD, the normal physiology, including pH, is altered [29]. Further, loading drugs into nanoparticles, whether polymeric or lipid-based, often results in low drug loadings ($<0.1 - 6\%$) [17, 30, 31], meaning that the bulk of the administered material is polymer or other excipients. In contrast, the NS approach that we describe here is composed of pure budesonide milled down to nano-size and coated with FDA generally regarded as safe (GRAS) Pluronic F127. However, despite the potential limitations of orally administered nanoparticles for treating disease in the distal colorectum, it is worth exploring whether oral nanoparticle formulations can be used in combination with NS enemas to further improve treatment efficacy.

Micro- and nanosuspensions are fluid formulations that contain water insoluble drugs that have been processed through bottom-up or top-down methods to form semi-stable suspensions of amorphous or crystalline drug particles [32, 33]. The approach is intended to

increase the surface area-to-volume ratio for improved dissolution, and has been thoroughly explored and developed to increase oral bioavailability of poorly bioavailable drugs [32, 34]. Micronization is also a strategy employed for clinically available enema formulations (budesonide and mesalamine) for local treatment of ulcerative colitis. However, large microparticulates are unlikely to penetrate the colorectal mucus barrier and be delivered to the affected tissues, suggesting that NS may be more efficacious for IBD enema therapies [9]. Further, because surface area scales by r^2 , a 10-fold decrease in particle size leads to a 100-fold increase in surface area for improved dissolution of poorly soluble drugs. Prior descriptions of budesonide NS include the use of a high-pressure homogenization apparatus to produce large scale batches (300 mL) for pulmonary administration, though the particle size achieved was 500–600 nm [35]. In another report, budesonide NS were produced via a bench top wet milling method using glass beads [36]. However, the method required 24 h of milling and resulted in a budesonide NS with ~400 nm average particulate size. In contrast, we describe here another version of modified wet milling using commercially available tissue homogenization equipment (TissueLyser LT) and inert zirconium oxide beads that enables transformation of budesonide in to NS formulation of the appropriate size (~225 nm) and surface characteristics (Pluronic F127 coated, -2 mV) for effective mucus penetration and improved colorectal distribution [9, 22, 23].

Intestinal inflammation leads to mucus hypersecretion and a reduction in barrier function, which has been demonstrated to have an impact on particle distribution and accumulation. We previously observed a reduction in steric barrier properties in the TNBS-induced colitis model, though adhesive interactions with mucoadhesive nanoparticles were maintained [9]. Others have similarly found increased accumulation of orally administered nanoparticles in inflamed areas of the intestines, presumably due to increased mucus production and a thicker mucus layer, which facilitates particle entrapment and adhesion [37]. However, there have been conflicting reports for how size impacts accumulation of particles at the inflamed mucosa; in several reports using animal models, smaller particle size lead to increased accumulation [37–39], whereas a clinical study suggested that microparticles accumulated more in areas of severe disease with ulcerous lesions compared to nanoparticles [40]. Another study using mucosal biopsies from IBD patients found that polyethylene glycol (PEG)-coated poly(lactic-co-glycolic acid) (PLGA) nanoparticles demonstrated increased penetration and translocation compared to mucoadhesive chitosan-coated PLGA nanoparticles [41]. Of note, the surface properties and core composition of the particles used in the aforementioned studies varied, and thus, more work is needed to fully elucidate the impact of particle size, surface properties, and particle composition on particle uptake and drug delivery in IBD patients [37]. Further, the increased accumulation of particles, particularly mucoadhesive particles, at inflamed mucosa makes determining discrete drug levels in colorectal cells and tissue technically challenging. The adherent mucus layer may be particularly challenging to remove [42], and differences in the efficiency of removing various sized particles by extensive washing [39] could be a result of differences in penetration depth or differences in cell uptake, but the reasons are unclear. Thus, it is particularly challenging to remove the luminal contents to measure drug absorption into tissue with drug-loaded particulate formulations in preclinical IBD models, and thus, particle localization studies and/or efficacy studies are the norm for assessing drug delivery

efficiency [16, 17, 30, 43]. Here, since the budesonide nano- and microsuspensions were composed only of budesonide and a physically associated surface coating material (F127 and PVP, respectively), it would be technically challenging to fluorescently label a component of the formulations without significantly impacting the stability and/or the physicochemical properties of the formulations. Thus, we used fluorescent polystyrene particles with the same respective surface coatings as model probes for assessing particle distribution and accumulation in the inflamed colons of mice with TNBS-induced colitis. We observed improved epithelial distribution and penetration of inflamed mucosa with the 200 nm/2% F127 particles compared to 2 μ m/1% PVP particles, which is likely due to both the smaller size and the muco-inert surface coating. This hypothesis was further supported by observing limited distribution and penetration of both 200 nm/1% PVP particles, suggesting that PVP coatings lead to mucoadhesion, and (ii) 2 μ m/2% F127 particles, suggesting that 2 μ m particles, even with muco-inert coatings, are too large to penetrate through the mucus and ulcerated tissue (steric trapping).

We employed the commonly used TNBS-induced colitis model for comparing the efficacy of our budesonide NS and MS enema formulations. Of note, the TNBS model has more immunological similarities with human CD (T_H1 driven) than with UC (T_H2 driven), though in humans, inflammation and mucosal damage in CD is not restricted to the colorectum like in UC [44]. However, the TNBS model is an attractive option for testing enema formulations, because the disease is reproducible and localized to the distal colorectum. Given that enemas are not expected to reach past the splenic flexure, enemas would not be expected to reach diffuse disease, particularly in the small intestine, which is more characteristic of CD. Furthermore, given the general anti-inflammatory properties of steroids like budesonide, it is not surprising that budesonide has been demonstrated to have efficacy in both the dextran sodium sulfate (DSS)-induced IBD model and the TNBS-induced models of IBD. However, given the diffused nature of the damage induced by orally administered DSS, the model is less optimal for testing enema formulations. Given our goal to compare our muco-inert NS formulation to a clinically-relevant budesonide MS, the TNBS model was appropriate for demonstrating improved efficacy for the NS. Our studies using the TNBS-induced colitis model showed that although both budesonide formulations led to a recovery in weight loss, the budesonide NS was more effective in reducing colon inflammation and normalizing tissue weight. Better distribution and penetration of muco-inert budesonide NS into the inflamed colon tissue could be responsible for the improved local effect. Histopathological studies also showed that only the mucus-penetrating budesonide NS enema could significantly ameliorate microscopic symptoms of IBD.

We also sought to confirm the efficacy of budesonide MS and NS formulations by analyzing immune cells in the colonic LP obtained from treated mice. Indeed, we found that local administration of both budesonide MS and NS significantly reduced the absolute number of viable colonic CD45+Ly6G+ cells (neutrophils) in mice with TNBS-induced colitis. Glucocorticoids like budesonide can promote the resolution of inflammation, and restore homeostasis, by promoting a wound-healing and anti-inflammatory activity in macrophages, inducing neutrophil and T cell apoptosis and promoting the removal of apoptotic cells [45, 46]. Moreover, it has been shown that glucocorticoids can induce the expression and secretion of Annexin-1, which can induce apoptosis of neutrophils at the site of

inflammation [47]. Thus, the reduction in neutrophils observed with budesonide MS or NS treatment could be a result of the apoptotic effect of glucocorticoids such as budesonide on neutrophils in the inflamed colon. Macrophages have been implicated in the pathogenesis of a variety of chronic and autoimmune diseases, including IBD [48, 49]. The classically activated macrophages are key producers of many cytokines (e.g. IL-1 β , TNF α and IL-6) and reactive metabolites of oxygen and nitrogen (e.g. nitric oxide) that have been implicated in the development of IBD [48, 49]. Macrophages, together with neutrophils, may contribute to intestinal damage by releasing reactive metabolites of oxygen and nitrogen [50]. It was previously shown that Ly6C^{high} monocytes are recruited into the inflamed gut to become the dominant inflammatory mononuclear cell type in the LP during acute colitis [21]. We found that only budesonide NS enema treatment could significantly reduce the number of viable colonic infiltrating monocytes (Ly6C^{high}CD11b+) in mice with TNBS-induced colitis, and to significantly reduce the production of inflammatory cytokines from CD11b+ cells. It is likely that the increased effect of the budesonide NS on reducing local inflammation led to the significant improvement in tissue histology observed in this acute TNBS-induced colitis model.

Finally, the mucus-penetrating budesonide NS enema formulation described here is composed of all FDA GRAS ingredients without chemical modification. The formulation approach uses similar principles as the MPP technology in development by Kala Pharmaceuticals that has already proven successful in Phase II and III clinical trials for ocular indications. There are several potential approaches to move forward with development of the budesonide enema formulation, which could be marketed as a stable, ready-to-use suspension like various mesalamine formulations (e.g. Rowasa®). Alternatively, taking a similar approach to budesonide enemas like Entocort®, a dispersible powder tablet could be sold alongside an enema bottle containing sterile vehicle and mixed by the user at the time of application. Future studies will include exploring the feasibility of both approaches for designing a budesonide NS product that can be reproducibly manufactured.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank BASF Inc. for providing free samples of Pluronic F127, the JHMI animal husbandry staff, the JHMI Reference Histology lab, the Wilmer Microscopy and Imaging Core Facility (MICF) funded by NIH grant P30EY001765, and the Drug Analysis Unit in the JHU Institute for Clinical and Translational Research (ICTR) funded in part by UL1 TR001079 from the National Center for Advancing Translational Sciences (NCATS) a component of the National Institutes of Health (NIH), and NIH Roadmap for Medical Research. This work was supported by NIH grants R21/R33AI079740, R01DK107806, and U19AI113127. We thank Yumin Oh, PhD, Seungwoo Chung, PhD, Kevin Liaw, and Karina Negron for helping with the preparation of the figures. We also thank Warren Strober and Ivan Fuss for helpful discussion and reading of the manuscript.

References

- [1]. Matricon J, Immunopathogenesis of inflammatory bowel disease, *M S-Med Sci* 26(4) (2010) 405–410.

- [2]. Campieri M, Ferguson A, Doe W, Persson T, Nilsson LG, Malchow H, Prantera C, Mani V, OMorain C, Selby W, Pallone F, diPietralata MM, Sjobahl R, Florin T, Smith P, Bianchi P, Lofberg R, Rutgeerts P, Smallwood R, Lamers HW, TasmanJones C, Hunter JO, Hodgson H, Danielsson A, Lee FI, Piacitelli G, Giovanni S, Ellis A, Weir DG, Oral budesonide is as effective as oral prednisolone in active Crohn's disease, *Gut* 41(2) (1997) 209–214. [PubMed: 9301500]
- [3]. O'Donnell S, O'Morain CA, Therapeutic benefits of budesonide in gastroenterology, *Therapeutic advances in chronic disease* 1(4) (2010) 177–86. [PubMed: 23251737]
- [4]. Cohen R, Advantages in IBD: Current Developments in the Treatment of Inflammatory Bowel Diseases, *Gastroenterology & Hepatology* 6(5) (2010) 309–16. [PubMed: 20567586]
- [5]. Ham M, Moss AC, Mesalamine in the treatment and maintenance of remission of ulcerative colitis, *Expert review of clinical pharmacology* 5(2) (2012) 113–23. [PubMed: 22390554]
- [6]. Sandborn WJ, Bosworth B, Zakko S, Gordon GL, Clemmons DR, Golden PL, Roller RL, Yu J, Barrett AC, Bortey E, Paterson C, Forbes WP, Budesonide foam induces remission in patients with mild to moderate ulcerative proctitis and ulcerative proctosigmoiditis, *Gastroenterology* 148(4) (2015) 740–750 e2. [PubMed: 25644096]
- [7]. Hanauer SB, Robinson M, Pruitt R, Lazenby AJ, Persson T, Nilsson LG, Walton-Bowen K, Haskell LP, Levine JG, Budesonide enema for the treatment of active, distal ulcerative colitis and proctitis: a dose-ranging study. U.S. Budesonide enema study group, *Gastroenterology* 115(3) (1998) 525–32. [PubMed: 9721148]
- [8]. Ensign LM, Cone R, Hanes J, Oral drug delivery with polymeric nanoparticles: the gastrointestinal mucus barriers, *Advanced drug delivery reviews* 64(6) (2012) 557–70. [PubMed: 22212900]
- [9]. Maisel K, Ensign L, Reddy M, Cone R, Hanes J, Effect of surface chemistry on nanoparticle interaction with gastrointestinal mucus and distribution in the gastrointestinal tract following oral and rectal administration in the mouse, *Journal of controlled release : official journal of the Controlled Release Society* 197C (2014) 48–57.
- [10]. Dahlstrom K, Edsbacker S, Kallen A, Rectal pharmacokinetics of budesonide, *European journal of clinical pharmacology* 49(4) (1996) 293–8. [PubMed: 8857075]
- [11]. Otten MH, De Haas G, Van den Ende R, Colonic spread of 5-ASA enemas in healthy individuals, with a comparison of their physical and chemical characteristics, *Alimentary pharmacology & therapeutics* 11(4) (1997) 693–7. [PubMed: 9305477]
- [12]. Ensign LM, Henning A, Schneider CS, Maisel K, Wang YY, Porosoff MD, Cone R, Hanes J, Ex vivo characterization of particle transport in mucus secretions coating freshly excised mucosal tissues, *Molecular pharmaceutics* 10(6) (2013) 2176–82. [PubMed: 23617606]
- [13]. Maisel K, Chattopadhyay S, Moench T, Hendrix C, Cone R, Ensign LM, Hanes J, Enema ion compositions for enhancing colorectal drug delivery, *Journal of controlled release : official journal of the Controlled Release Society* 209 (2015) 280–7. [PubMed: 25937321]
- [14]. Ensign LM, Tang BC, Wang YY, Tse TA, Hoen T, Cone R, Hanes J, Mucus-penetrating nanoparticles for vaginal drug delivery protect against herpes simplex virus, *Sci Transl Med* 4(138) (2012) 138ra79.
- [15]. Ensign LM, Hoen TE, Maisel K, Cone RA, Hanes JS, Enhanced vaginal drug delivery through the use of hypotonic formulations that induce fluid uptake, *Biomaterials* 34(28) (2013) 6922–9. [PubMed: 23769419]
- [16]. Duman U, Yilmazlar A, Ozturk E, Aker S, Sarandol E, Yilmazlar T, Anti-inflammatory efficiency of levobupivacaine in an experimental colitis model, *World J Gastroentero* 16(20) (2010) 2537–2541.
- [17]. Ali H, Weigmann B, Neurath MF, Collnot EM, Windbergs M, Lehr CM, Budesonide loaded nanoparticles with pH-sensitive coating for improved mucosal targeting in mouse models of inflammatory bowel diseases, *Journal of controlled release : official journal of the Controlled Release Society* 183 (2014) 167–77. [PubMed: 24685705]
- [18]. Kremer B, Mariman R, van Erk M, Lagerweij T, Nagelkerken L, Temporal colonic gene expression profiling in the recurrent colitis model identifies early and chronic inflammatory processes, *PloS one* 7(11) (2012) e50388. [PubMed: 23226271]
- [19]. Wirtz S, Neufert C, Weigmann B, Neurath MF, Chemically induced mouse models of intestinal inflammation, *Nat Protoc* 2(3) (2007) 541–6. [PubMed: 17406617]

- [20]. Geem D, Medina-Contreras O, Kim W, Huang CS, Denning TL, Isolation and characterization of dendritic cells and macrophages from the mouse intestine, *J Vis Exp* (63) (2012) e4040. [PubMed: 22644046]
- [21]. Zigmund E, Varol C, Farache J, Elmaliah E, Satpathy AT, Friedlander G, Mack M, Shpigel N, Boneca IG, Murphy KM, Shakhar G, Halpern Z, Jung S, Ly6C hi monocytes in the inflamed colon give rise to proinflammatory effector cells and migratory antigen-presenting cells, *Immunity* 37(6) (2012) 1076–90. [PubMed: 23219392]
- [22]. Yang M, Lai SK, Wang YY, Zhong W, Happe C, Zhang M, Fu J, Hanes J, Biodegradable nanoparticles composed entirely of safe materials that rapidly penetrate human mucus, *Angewandte Chemie* 50(11) (2011) 2597–600. [PubMed: 21370345]
- [23]. Yu T, Chisholm J, Choi WJ, Anonuevo A, Pulicare S, Zhong WX, Chen MM, Fridley C, Lai SK, Ensign LM, Suk JS, Hanes J, Mucus-Penetrating Nanosuspensions for Enhanced Delivery of Poorly Soluble Drugs to Mucosal Surfaces, *Adv Healthc Mater* 5(21) (2016) 2745–2750. [PubMed: 27717163]
- [24]. Probert CS, Dignass AU, Lindgren S, Oudkerk Pool M, Marteau P, Combined oral and rectal mesalazine for the treatment of mild-to-moderately active ulcerative colitis: rapid symptom resolution and improvements in quality of life, *Journal of Crohn's & colitis* 8(3) (2014) 200–7.
- [25]. Hua S, Marks E, Schneider JJ, Keely S, Advances in oral nano-delivery systems for colon targeted drug delivery in inflammatory bowel disease: selective targeting to diseased versus healthy tissue, *Nanomedicine : nanotechnology, biology, and medicine* 11(5) (2015) 1117–32.
- [26]. Lu L, Chen GX, Qiu YY, Li MW, Liu DH, Hu DH, Gu XJ, Xiao ZY, Nanoparticle-based oral delivery systems for colon targeting: principles and design strategies, *Sci Bull* 61(9) (2016) 670–681.
- [27]. McConnell EL, Short MD, Basit AW, An in vivo comparison of intestinal pH and bacteria as physiological trigger mechanisms for colonic targeting in man, *Journal of controlled release : official journal of the Controlled Release Society* 130(2) (2008) 154–60. [PubMed: 18639950]
- [28]. Ibekwe VC, Fadda HM, McConnell EL, Khela MK, Evans DF, Basit AW, Interplay between intestinal pH, transit time and feed status on the in vivo performance of pH responsive ileo-colonic release systems, *Pharm Res-Dordr* 25(8) (2008) 1828–1835.
- [29]. Nugent SG, Kumar D, Rampton DS, Evans DF, Intestinal luminal pH in inflammatory bowel disease: possible determinants and implications for therapy with aminosaliculates and other drugs, *Gut* 48(4) (2001) 571–577. [PubMed: 11247905]
- [30]. Beloqui A, Coco R, Alhouayek M, Solinis MA, Rodriguez-Gascon A, Muccioli GG, Preat V, Budesonide-loaded nanostructured lipid carriers reduce inflammation in murine DSS-induced colitis, *International journal of pharmaceutics* 454(2) (2013) 775–83. [PubMed: 23694806]
- [31]. Naeem M, Choi M, Cao J, Lee Y, Ikram M, Yoon S, Lee J, Moon HR, Kim MS, Jung Y, Yoo JW, Colon-targeted delivery of budesonide using dual pH- and time-dependent polymeric nanoparticles for colitis therapy, *Drug design, development and therapy* 9 (2015) 3789–99.
- [32]. Rabinow BE, Nanosuspensions in drug delivery, *Nature reviews. Drug discovery* 3(9) (2004) 785–96. [PubMed: 15340388]
- [33]. Verma S, Gokhale R, Burgess DJ, A comparative study of top-down and bottom-up approaches for the preparation of micro/nanosuspensions, *International journal of pharmaceutics* 380(1–2) (2009) 216–22. [PubMed: 19596059]
- [34]. Gao L, Liu G, Ma J, Wang X, Zhou L, Li X, Wang F, Application of drug nanocrystal technologies on oral drug delivery of poorly soluble drugs, *Pharm Res* 30(2) (2013) 307–24. [PubMed: 23073665]
- [35]. Jacobs C, Muller RH, Production and characterization of a budesonide nanosuspension for pulmonary administration, *Pharm Res* 19(2) (2002) 189–94. [PubMed: 11883646]
- [36]. Yang JZ, Young AL, Chiang PC, Thurston A, Pretzer DK, Fluticasone and budesonide nanosuspensions for pulmonary delivery: preparation, characterization, and pharmacokinetic studies, *Journal of pharmaceutical sciences* 97(11) (2008) 4869–78. [PubMed: 18351635]
- [37]. Youshia J, Lamprecht A, Size-dependent nanoparticulate drug delivery in inflammatory bowel diseases, *Expert Opin Drug Deliv* 13(2) (2016) 281–94. [PubMed: 26637060]

- [38]. Vong LB, Tomita T, Yoshitomi T, Matsui H, Nagasaki Y, An orally administered redox nanoparticle that accumulates in the colonic mucosa and reduces colitis in mice, *Gastroenterology* 143(4) (2012) 1027–36 e3. [PubMed: 22771506]
- [39]. Lamprecht A, Schafer U, Lehr CM, Size-dependent bioadhesion of micro- and nanoparticulate carriers to the inflamed colonic mucosa, *Pharm Res* 18(6) (2001) 788–93. [PubMed: 11474782]
- [40]. Schmidt C, Lautenschlaeger C, Collnot EM, Schumann M, Bojarski C, Schulzke JD, Lehr CM, Stallmach A, Nano- and microscaled particles for drug targeting to inflamed intestinal mucosa: a first in vivo study in human patients, *Journal of controlled release : official journal of the Controlled Release Society* 165(2) (2013) 139–45. [PubMed: 23127508]
- [41]. Lautenschlager C, Schmidt C, Lehr CM, Fischer D, Stallmach A, PEG-functionalized microparticles selectively target inflamed mucosa in inflammatory bowel disease, *Eur J Pharm Biopharm* 85(3 Pt A) (2013) 578–86. [PubMed: 24084650]
- [42]. Atuma C, Strugala V, Allen A, Holm L, The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo, *Am J Physiol Gastrointest Liver Physiol* 280(5) (2001) G922–9. [PubMed: 11292601]
- [43]. Ali H, Weigmann B, Collnot EM, Khan SA, Windbergs M, Lehr CM, Budesonide Loaded PLGA Nanoparticles for Targeting the Inflamed Intestinal Mucosa--Pharmaceutical Characterization and Fluorescence Imaging, *Pharm Res* 33(5) (2016) 1085–92. [PubMed: 26718953]
- [44]. Scheiffele F, Fuss IJ, Induction of TNBS colitis in mice, *Current protocols in immunology / edited by Coligan John E. ... [et al.] Chapter 15* (2002) Unit 15 19.
- [45]. Rhen T, Cidlowski JA, Antiinflammatory action of glucocorticoids--new mechanisms for old drugs, *N Engl J Med* 353(16) (2005) 1711–23. [PubMed: 16236742]
- [46]. Busillo JM, Cidlowski JA, The five Rs of glucocorticoid action during inflammation: ready, reinforce, repress, resolve, and restore, *Trends Endocrinol Metab* 24(3) (2013) 109–19. [PubMed: 23312823]
- [47]. Baschant U, Tuckermann J, The role of the glucocorticoid receptor in inflammation and immunity, *J Steroid Biochem Mol Biol* 120(2–3) (2010) 69–75. [PubMed: 20346397]
- [48]. Sartor RB, Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis, *Nat Clin Pract Gastroenterol Hepatol* 3(7) (2006) 390–407. [PubMed: 16819502]
- [49]. Fujino S, Andoh A, Bamba S, Ogawa A, Hata K, Araki Y, Bamba T, Fujiyama Y, Increased expression of interleukin 17 in inflammatory bowel disease, *Gut* 52(1) (2003) 65–70. [PubMed: 12477762]
- [50]. Kriegelstein CF, Cerwinka WH, Laroux FS, Salter JW, Russell JM, Schuermann G, Grisham MB, Ross CR, Granger DN, Regulation of murine intestinal inflammation by reactive metabolites of oxygen and nitrogen: divergent roles of superoxide and nitric oxide, *J Exp Med* 194(9) (2001) 1207–18. [PubMed: 11696587]

Figure 1.

Distribution of model fluorescent (A) PVP-coated 2 μm PS particles (surrogate for clinical enema) and (B) Pluronic F127-coated 200 nm PS particles in the colons of mice with TNBS-induced colitis. F127-coated 200 nm PS particles appeared to penetrate into the damaged colorectal tissue, whereas 2 μm PS particles were largely present in the colorectal lumen. In contrast, both (C) PVP-coated 2 μm PS particles and (D) F127-coated 200 nm PS particles were excluded from intact colorectal tissue in healthy mice, while more uniform mucosal distribution was evident for the 200 nm particles in the healthy mouse colorectum. Images are representative of $n = 3$ mice.

Figure 2: Optimization, characterization and *in vitro* anti-inflammatory activity of budesonide NS.

Various parameters of wet-milling process such as (A) size of zirconium oxide beads, (B) budesonide to bead mass ratio (C) Pluronic F127 concentration and (D) wet-milling time were optimized to obtain Pluronic F127 coated budesonide NS in the size range appropriate for penetration of mouse colorectal mucus (~222 nm and polydispersity index = 0.2). Transmission electron micrograph of budesonide NS (Scale bar = 500 nm). (F) *In vitro* anti-inflammatory activity of budesonide NS was evaluated to assess the impact of wet-milling process on drug activity. RAW264.7 cells were activated with LPS (0.5 μ g/ml) for 5 hours in the presence or absence of 1 μ M of budesonide solution (SOL), MS, or NS and the production of the pro-form of IL-1 β from CD11b+ RAW264.7 cells was measured using flow cytometry. Budesonide SOL, MS and NS were able to reduce the production of the pro-form of IL-1 β in LPS activated RAW264.7 cells compared to control (CTRL). Data are expressed as mean $\pm$ SEM (n = 3). * p < 0.05, ** p < 0.01.

Figure 3: Treatment with the budesonide NS enema provides significant reductions in macroscopic and microscopic symptoms in mice with TNBS-induced colitis
 (A) Mice treated with budesonide MS (TNBS + MS) or NS (TNBS + NS) enema showed a significant recovery in body weight compared to untreated (TNBS) animals. (B) Only the budesonide NS treated animals showed significantly lower colon weight compared to untreated animals (n = 15 per group). (C) H&E stained colon sections revealed local alterations of cellular structure, ulcerations, neutrophil infiltration, and tissue damage in untreated control and MS treated mice, whereas treatment with the budesonide NS provided amelioration of microscopic IBD symptoms. (D) Quantitative histopathological scoring based on mucosal damage, destruction of cellular architecture, infiltration and epithelial regeneration, and percent colon involved demonstrated a significant reduction in disease with budesonide NS enema treatment compared to untreated mice and MS treated mice (n = 5 per group). Data are expressed as mean $\pm$ SEM; * p < 0.05, ** p < 0.01.

Figure 4: Only budesonide NS significantly lowered the number of viable inflammatory macrophages, a cell type implicated in the pathogenesis of IBD, in the colon lamina propria. Treatment with budesonide MS and NS significantly reduced the number of (A) infiltrating neutrophils (Ly6G⁺/CD45⁺ cells; n = 5 per group), and (B) only budesonide NS showed reduction in inflammatory macrophages (Ly6C^{high}/CD11b⁺/CD45⁺ cells; n = 7 per group) and (C) reduction in the production of the pro-form of inflammatory cytokine IL-1β from the CD11b⁺ cells (n = 4 per group). Data are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.